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Megalin-Mediated Oligonucleotide Trafficking for Breast Cancer Chemosensitization

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13. SUPPLEMENTARY NOTES

14. ABSTRACT The delivery of the large and charged oligonucleotides such as siRNA to cancer cells is a highly inefficient process. Megalin is an endocytotic surface receptor highly expressed in breast cancer cells that can be exploited for enhanced cellular siRNA uptake. The goals of this study are to develop the necessary tools and validate a novel strategy to target megalin for improved delivery of anticancer siRNA. Lipid nanocarriers (LNC) for delivery of fluorescent siRNA or siRNA targeting clusterin were prepared. The LNC system was shown capable of binding the megalin targeting substrate ApoE at 91.2 ± 4.8% efficiency. The ApoE-binding on LNC was shown highly stable. Only 8.5 ± 3.3% of the bound ApoE came off after 48 hr incubation at 37 ℃. The encapsulation efficiency of siRNA was 75.2 ±6.1%. After siRNA encapsulation, the size of ApoE-LNC was measured at 241 nm (PDI = 0.168). This system demonstrated very low non-specific toxicity as measured by trypan blue assay. The uptake of fluorescent LNC into MCF-7 cells was visibly increased several folds after ApoE modification, showing the value of megalin targeting for siRNA delivery. Our early data have also demonstrated strong megalin and clusterin expression in MCF-7 cells, indirectly supporting the potential value of the proposed targeted strategy. Future studies will further confirm the correlation between ApoE coating of a siRNA carrier and megalin expression and clusterin knockdown.

15. SUBJECT TERMS
Targeted delivery, nanomedicine, siRNA, megalin, clusterin, breast cancer cell, drug resistance

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A. INTRODUCTION

Megalin is an efficient endocytotic-receptor significantly expressed in breast cancer cells so it may serve as a targetable gateway for cancer therapeutics trafficking [1]. High megalin expression may reflect the level of chemoresistance of the cancer cells [2], while clusterin is known to cause chemoresistance in breast cancer [3]. Both megalin and clusterin tend to be upregulated by breast cancer chemotherapy such as paclitaxel treatment [4]. Targeting megalin for oligonucleotide-based (e.g. small-interfering RNA or siRNA) clusterin knockout therefore may represent a more coherent and effective resistance-targeting strategy. The key hypothesis here is that coating a siRNA carrier such as lipid-based nanocarrier with megalin-targeting moieties such as apolipoprotein E (Apo-E) [5] will enhance siRNA delivery into breast cancer cells which reportedly express high megalin level. Accordingly, two aims were proposed as follows: (a) to determine the feasibility of using megalin-targeted strategy to improve the delivery of oligonucleotide into breast cancer cells; (b) to study the effect of megalin-targeted delivery on the biological activities of anti-clusterin oligonucleotides in breast cancer cells. Overall, after one year of study from Jul 2009 to Jun 2010, our group has nearly completed the first aim. The ApoE-modified carrier platform has been properly developed and characterized, and improved uptakes of the ApoE-carriers were observed. We have also collected early data in support of the studies of the second aim.

B. BODY

B.1. Task 1: Feasibility studies of megalin-targeted strategy for improvement of oligonucleotide delivery to breast cancer

Subtask 1a. Prepare and characterize megalin-targeting oligonucleotide carrier,

• **Preparation of core carrier platform for megalin-targeted delivery of siRNA:** The core lipid nanocarrier (LNC) for studying of megalin targeting was prepared as described by Wong et al [6] with modest modifications for ApoE coating and clusterin-siRNA encapsulation. Briefly, trimyristin and oleic acid were mixed at 90:10 w/w ratio, warmed to 65 ^{o}C. 2-3% polysorbate-80 is added and the mixture is sonicated, extruded through 200 nm polycarbonate filter, and cooled to 25 ^{o}C to form the nanocarrier. 2.5% DOPC and 0.5-1% fluorescent lipids (1,2-dioleoyl-sn-glycero-3'phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) or FITC-equivalent) were typically added to the lipid mixture to improve stability and enable detection by fluorescence. For siRNA encapsulation fluorescent-tagged siRNA or clusterin-targeting siRNA was included in the above mixture together with poly-arginine (+/- charge ratio = 2:1). Unencapsulated siRNA molecules were removed by passing through columns containing Sephadex G-25.

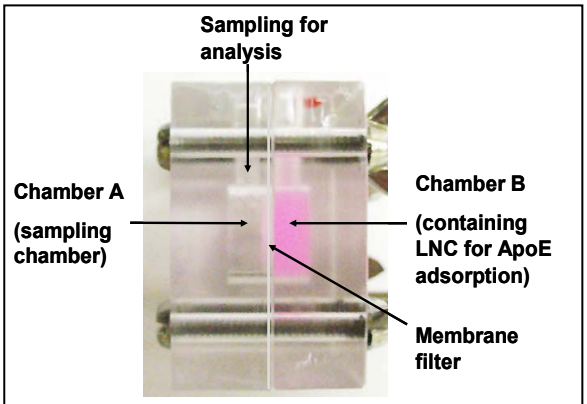

Fig. 1. Diffusion cell setup used for protein adsorption study to determine ApoE binding on LNC. LNC tagged with rhodamine-conjugated DOPC lipid (so pink in color)

• **Surface coating of LNC with ApoE for megalin-targeting**: The above core LNC was coated with Apo-E and the mass balance analysis was performed as follows. ApoE (100 μg) and LNC (1 mg) suspending in colorless RPMI-1640 medium were introduced to each of the two chambers of a dialysis/diffusion cell, respectively (**Fig. 1**). The two chambers were separated by a polycarbonate filter (50 nm pore size). This membrane filter kept the LNC in chamber B but allowed the ApoE to freely diffuse across it when it was not bounding on LNC. LNC also included a small quantity of pink-colored rhodamine-conjugated DOPC lipids to allow detection of membrane leakage (as seen in Fig. 1, leakage did not occur). Free, unbound ApoE was quantified using Pierce micro-BCA assay kit using BSA as a standard. We showed that:

(a) LNC were able to bind to 91.2 ± 4.8% of the ApoE added (assuming % bound protein = 100 – [2 x unbound protein], unbound ApoE) after 24 hr incubation at 37 ^{o}C on a rocking platform, and no further increase upon longer incubation;

(b) At the end of the above study, the content in chamber-A was emptied, chamber-A rinsed twice and then refilled with fresh medium, and the diffusion cell re-incubated at 37 ^{o}C for additional 48 hr. Only 8.5 ± 3.3% of the bound ApoE came off from the LNC in chamber B and back-diffused into chamber A.

(c) When the final ApoE-LNC suspension was passed through a 0.05 μm filter, no detectable amount of protein was found in the filtrate.

Overall, the results showed that LNC can attach ApoE to form a stable megalin-targeting LNC platform.

• **Characterization for size, charge, encapsulation efficiency:** Particle sizes and zeta potentials were measured by photon correlation spectroscopy (PCS). ApoE coating moderately shifted the particle size upward as shown in **Fig. 2** in the next page.

(a) The mean diameter of LNC was increased from 140 nm (PDI=0.13) to 178 nm (PDI=0.142) after ApoE-coating.

(b) The zeta potentials did not significantly change (without coating: -20.8 mV; with ApoE coating: -22.0 mV) after ApoE coating.

(c) The encapsulation efficiency of siRNA was 75.2 ±6.1% (n=3 independent samples). After siRNA encapsulation, the size of ApoE-LNC increased to 241 nm (PDI = 0.168).

The data showed that the size ranges of various carriers were generally adequate for cell internalization and distribution, and a high siRNA encapsulation could be achieved with LNC to perform the studies.

- **ApoE-coated carriers show low non-specific, acute toxicity to non-cancer and cancer cells:** Studies have shown that the prototypical lipid carriers are highly biocompatible [7]. To confirm this advantage in LNC and apoE-coated LNC, trypan blue exclusion assay was conducted using MCF-7 breast cancer cell line and the non-cancerous MCF-10A mammary epithelial cell line. As in **Fig. 3**, after 5 hr treatment, both LNC's (non-coated: NC and apoE-coated) did not exhibit significant toxicity at ≤ 1 %w/v concentration in cancer or non-cancer cells. Results show that the intrinsic toxicity of all LNC should not be a confounding factor in our studies, as the highest LNC concentration used did not exceed 0.02%.

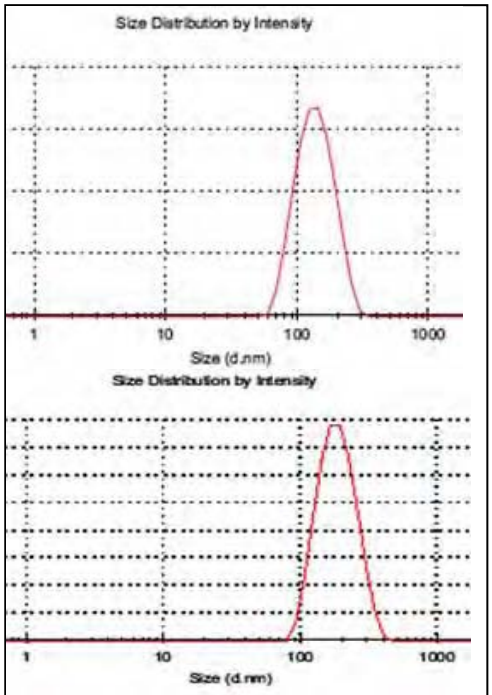

Fig. 2. PCS measurement of the size of (top) uncoated LNC; (bottom) ApoE-coated LNC.

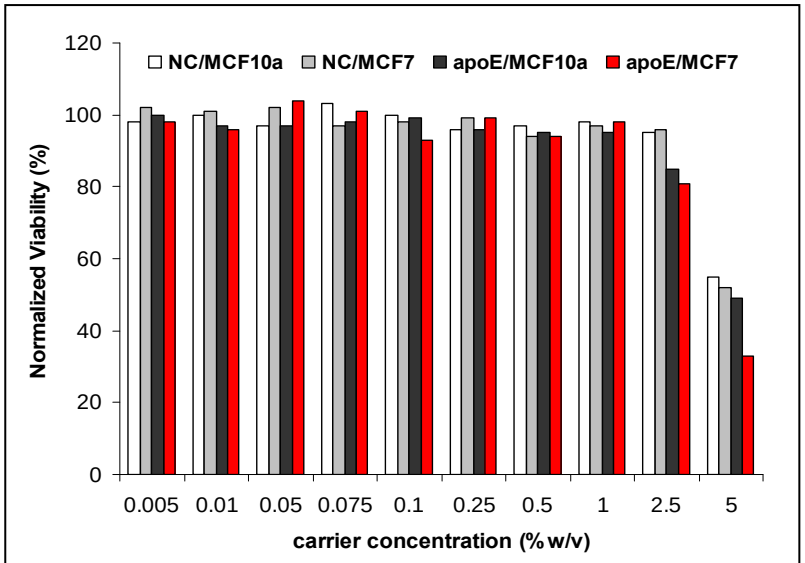

Fig. 3. Trypan blue exclusion assay to evaluate the acute, non-specific toxicity of ApoE-coated carriers; NC = non-coated carrier; apoE = ApoE coated carriers. Results normalized vs vehicle control (n=3)

Subtask 1b. Evaluate the effect of megalin-targeting on cellular uptake of oligonucleotides/ carriers

- **Improved uptake of ApoE-LNC into MCF-7 cells.**

With the ApoE coating on LNC and megalin expression in MCF-7 confirmed, we study the effect of ApoE coating on cellular uptake for oligonucleotide trafficking. **Fig. 5** compares the uptake of fluorescent-labeled LNC with or without ApoE coating into MCF-7 cells 2 hr after cell-LNC interaction. The same brightness/exposure setting that allows detection of moderate level of uncoated LNC (left) results in strong (in fact, over-saturation of) fluorescence in the ApoE group (right). More

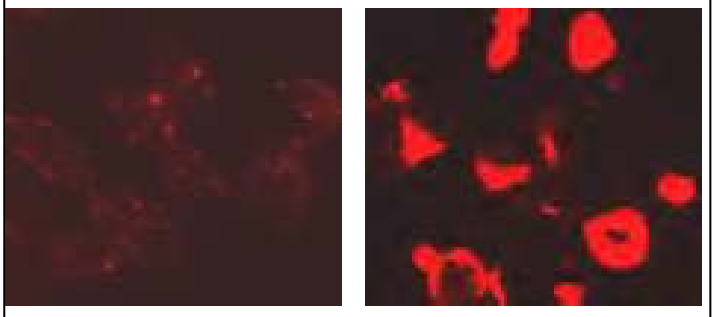

Fig. 5. Cellular uptake of labeled LNC into MCF-7 cells. Left: uncoated LNC; Right: ApoE-coated LNC.

optimization is required to generate better quality images, but the initial finding has already strongly indicated that the megalin-targeting strategy with ApoE coating can significantly improve the nanomedicine uptake by breast cancer cells.

Subtask 1c. Study the correlation between cellular megalin expression and oligonucleotide uptake

- **Western blot analysis confirms expression and up-regulation of megalin in MCF-7 after paclitaxel exposure**.

The aims of this study are to confirm that the main breast cancer cell line (MCF-7) we use actually expresses megalin as previously reported and evaluate if prior exposure to chemotherapy (paclitaxel) will up-regulate megalin. MCF-7 cells were exposed to paclitaxel or siRNA targeting megalin for 5 hr, rinsed and cultured in drug-free medium for 48 hr, and subjected to Western blot analysis. Results are shown in **Fig. 4**. The band intensities were normalized against the chemo-naïve (0nM paclitaxel) group. Overall, chemo-naïve MCF expressed megalin at a level comparable to the positive control (kidney lysate). The identity of megalin was confirmed by the visible knockdown by the megalin-targeting siRNA. Prior exposure to paclitaxel was found to enhance the megalin expression.

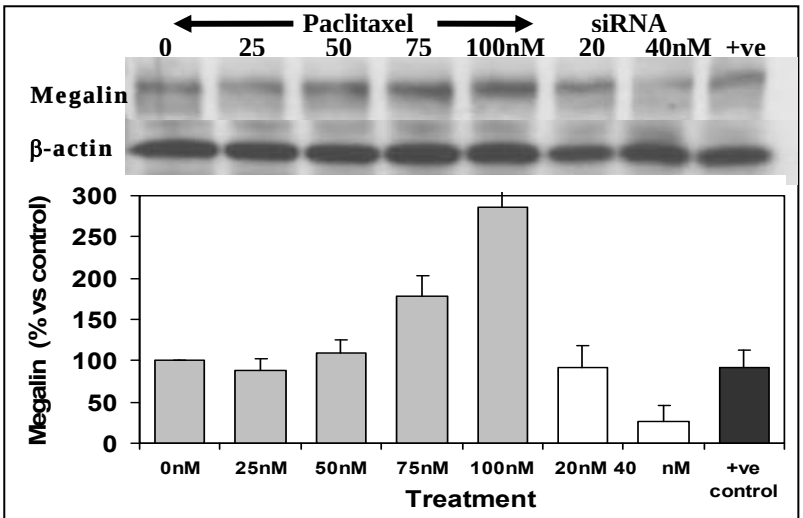

Fig. 4. Western blot analysis of megalin levels in MCF-7

In brief, the findings strongly indicated that the MCF-7 cell line is useful to test the megalin-targeting LNC. They also provided further evidence supporting that the proposed megalin-targeting strategy should work even better in chemotherapy treated cancers as a result of upregulated megalin expression.

It must be noted that because megalin is a protein with very high MW (600 kDa), the protein transfer process from the polyacrylamide gel to the PVDF membrane was highly inefficient (as indicated by strong staining of the protein leftover in the gel portion). In fact, we had technical difficulty to achieve significant protein transfer for this study, so the results here should be an under-estimate and it is difficult to establish a quantitative correlation between cellular megalin expression and siRNA/LNC uptake as proposed in subtask 1c. Therefore, we propose to use immunoprecipitation for this study. Our group has been currently testing the experimental conditions for this procedure.

B.2. Task 2: Evaluation of effect of megalin-targeted strategy on the therapeutic activities of anti-clusterin oligonucleotides

Subtask 2a. Evaluate changes in expression of clusterin and related pharmacodynamic parameters after targeted anti-clusterin treatments

- **Initial evaluation of siRNA mediated clusterin knockdown in breast cancer cells.**

Our proposed task 2 is to determine if the megalin-targeting strategy for siRNA delivery can improve the clusterin knockdown in breast cancer cells. In order to achieve this we need to first obtain the siRNA dose-response relationship using the standard gene-transfecting agent as a reference for future comparison. **Fig. 6** shows the clusterin knockdown effect of different concentrations of siRNA delivered by Lipofectamine-2000. Cells were treated with siRNA for 5 hr, rinsed and re-incubated in fresh medium for 48 hr. Western blot analysis was performed using antibody of clusterin. Because clusterin-α was targeted the combined bands of clusterin-α sub-unit (the lower band at around 38 kDa) and the full clusterin protein (the upper band around 55 kDa) were presented here. The result showed that significant knockdown of clusterin could be achieved with relatively low siRNA level (40 nM) in MCF-7 to ~ 44% of the baseline level. Further increase in the siRNA level to even up to 120 nM only provided modest improvement (to ~ 40% of the baseline). In TD-47 cell line, the clusterin expression was simply so strong that even at 120 nM siRNA the clusterin level was knocked down by 17% compared to the 0 nM group. Further studies will be required to obtain the optimal conditions for our proposed functionality studies.

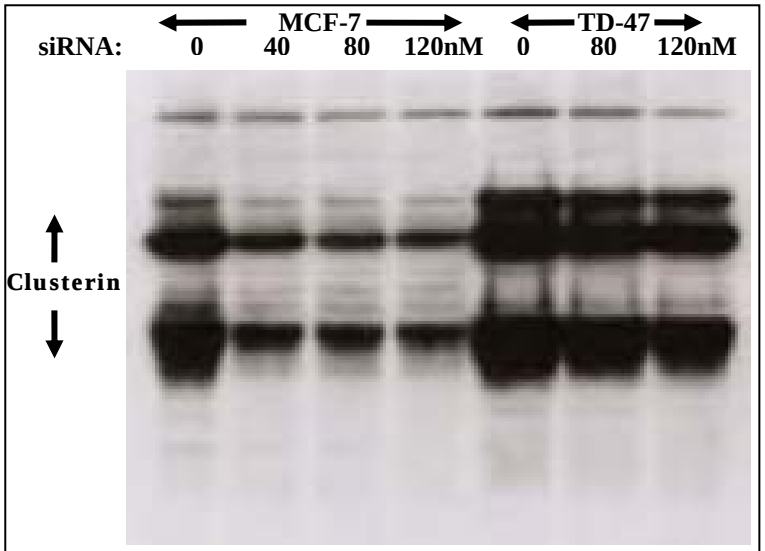

Fig. 6. Western blot analysis of clusterin in breast cancer

B.3. Future Studies.

In brief, we will complete the measurement of megalin expression in different cell lines using immunoprecipitation, thereby establish the more accurate and quantitative correlation with the uptake of ApoE-coated LNC encapsulating siRNA. With the targeting strategy fully validated, we will proceed to demonstate its benefit in improving the clusterin knockdown and chemosensitizaton effect.

C. KEY RESEARCH ACCOMPLISHMENTS

- Successfully prepared a lipid nanocarrier (LNC) system suitable for siRNA encapsulation and ApoE coating.
- Demonstrated stable coating of the megalin substrate ApoE on the LNC with high efficiency (>90%).
- Characterized the LNC systems with or without ApoE coating.
- Shown that the systems have low intrinsic toxicity to non-cancer breast epithelial cells (MCF-10A)
- Demonstrated substantial improved uptake into MCF-7 cells with the ApoE coating.
- Performed early studies confirming that breast cancer cell lines strongly express megalin and clusterin, and their levels could be up-regulated after paclitaxel exposure.

D. REPORTABLE OUTCOMES

A part of the data used for the development of LNC has led to the following international conference abstracts/posters:

1. Xue HY, Wong HL. Manipulation of lipid composition in nanostructured lipid devices for sustained, controlled intracellular small-interfering RNA release, Abstract 1505, AAPS Conference 2010.

2. Xue HY, Narvekar M, Wong HL. Shaping of intracellular kinetics of small-interfering RNA for extended, effective cancer chemo-RNAi-therapy, Abstract 1506, AAPS Conference 2010.

E. CONCLUSION

The study has successfully developed the carrier platform that is essential for the implementation of the megalin targeting strategy. This platform (i.e. ApoE-LNC) was shown to substantially improve the intracellular uptake rate into breast cancer cells in vitro. Further studies are undergoing to establish the correlation between megalin expression and siRNA trafficking by this ApoE-LNC system, and how this can lead to increased anticancer effect of the siRNA-mediated clusterin knockdown therapy.

F. REFERENCES

1. Rowling MJ, Kemmis CM, Taffany DA, Welsh J. Megalin-mediated endocytosis of vitamin D binding protein correlates with 25-hydroxycholecalciferol actions in human mammary cells J Nutr 136: 2754–2759, 2006.

2. Chlon TM, Taffany DA, Welsh J, Rowling MJ. Retinoids modulate expression of the endocytic partners megalin, cubilin, and disabled-2 and uptake of vitamin D-binding protein in human mammary cells. J Nutr 138:1323-8, 2008.

3. Redondo M, Tellez T, Roldan MJ, Serrano A, Garcia-Aranda M, Gleave ME, Hortas ML, Morell M. Anticlusterin treatment of breast cancer cells increases the sensitivities of chemotherapy and tamoxifen and counteracts the inhibitory action of dexamethasone on chemotherapy-induced cytotoxicity. Breast Cancer Res 9:R86, 2007.

4. Ammar H, Closset JL. Clusterin activates survival through the phosphatidylinositol3-kinase/Akt pathway. J Biol Chem 283:12851-12861, 2008.

5. Schwarz M, Spath L, Lux CA, Paprotka K, Torzewski M, Dersch K, Koch-Brandt C, Husmann M, Bhakdi S, Potential protective role of apoprotein J (clusterin) in atherogenesis: binding to enzymatically modified low-density lipoprotein reduces fatty acid-mediated cytotoxicity. Thrombosis & Haemostasis 100:110-8, 2008.

6. Xue HY, Wong HL. Manipulation of lipid composition in nanostructured lipid devices for sustained, controlled intracellular small-interfering RNA release, Abstract 1505, AAPS Conference 2010.

7. Muller, R.H., Maaben, S., Weyhers, H., Specht. F., and Lucks. J.S. Cytotoxicity of magnetite loaded polylactide, polylactide/glycolide particles and solid lipid nanoparticles (SLN). Int. J. Pharm. 138: 85-94, 1996.

Manipulation of lipid composition in nanostructured lipid devices for sustained, controlled intracellular small-interfering RNA release

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Purpose. Small-interfering RNAs (siRNA) have been known for their brief duration of activity and poor stability, which may offset their strong potential for therapeutic uses. Unlike conventional drug compounds, siRNA molecules that are released from their carriers do not enter cells by themselves efficiently. Sustained, controlled siRNA release is therefore best achieved in the intracellular compartment (e.g. an internalized carrier). This study focuses on developing the intracellular siRNA profile-shaping strategy for sustained RNA-interference by manipulating the enzymatic degradation behaviors of nanostructured lipid carriers.

Methods. Nanostructured carriers consisting of a triglyceride-based solid lipid framework incorporating liquid oil droplets were prepared and characterized by photon correlation spectroscopy and differential scanning calorimetry. The effects of various parameters on the degradation rates of the devices by lysosomal acid lipase (LAL) and siRNA release kinetics were spectrophotometrically monitored. Cancer cells were transfected with different carrier formulations encapsulating a non-silencing siRNA duplex (5'-AATTCTCCGAACGTG TCACGT-3') conjugated with fluorescent moiety and the intracellular siRNA kinetics were tracked by epifluorescence microscopy. Data were compared to the standard transfection agents.

Results. The nanostructured carriers were around 200 nm in diameter, which increased to the range of 250-300 nm with siRNA encapsulation. In LAL buffer which simulated lysosomal environment, it was shown that only the liquid oil content in the carrier significantly ($p<0.01$) affected the carrier degradation rates. Increased oil content led to faster carrier degradation by LAL. Parameters such as the type of triglyceride solid lipids and mixing of lipids did not affect this process. A similar trend was observed in the siRNA release profiles when siRNA-loaded carriers were LAL treated. Microscopy measurements showed 7 days and 5 days of intracellular release of fluorescent siRNA when the carrier contained 10% and 30% oil content, respectively, *versus* only 3 days when cells were transfected with DOTAP-cholesterol liposomes or a proprietary transfection agent.

Conclusions. By carefully manipulating the lipid composition, particularly the oil content in a nanostructured lipid-based carrier, sustained and controlled intracellular kinetics of siRNA can be conveniently achieved. This may expand the applicability of siRNA for experimental uses, and allow more optimal dosing and treatment in clinical settings.

Shaping of intracellular kinetics of small-interfering RNA for extended, effective cancer chemo-RNAi-therapy

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[1]Temple University School of Pharmacy

Purpose. The prospect of using small-interfering RNAs (siRNA) for cancer treatment is attractive because of their strong potency and good specificity for silencing selected oncogenic or drug-resistance pathways, e.g. survivin, by RNA-interference (RNAi) mechanism. However, the short duration of RNAi activity and risk of dose-related side effects may compromise their therapeutic value. Here we propose to implement the intracellular release profile-shaping strategy based on novel nanostructured siRNA carriers (NSC) to achieve extended and more effective RNAi-based chemosensitization.

Methods. Two NSC formulations containing different lipid compositions were prepared to encapsulate siRNA targeting survivin (surv-siRNA). These carriers have been demonstrated to result in different intracellular siRNA release profiles (fast-NSC: lasts 5 days, slow-NSC: > 7 days). Human prostate PC3 cancer cells were transfected with fast- or slow-NC carrying surv-siRNA. Western blot analysis was conducted to evaluate the survivin knockdown time-profiles, and viability assays (MTT and clonogenic) performed to evaluate the effective time-windows of RNAi-based chemosensitization of PC3 cells to docetaxel.

Results. Fast and slow NSC formulations were both able to knock down survivin to <25% of the original level. This confirms the functionality of the siRNA molecules encapsulated in these novel carriers. These NSC formulations led to longer durations of survivin knockdown compared to when the siRNA was delivered by DOTAP-cholesterol liposomes or a proprietary transfection agent. The duration of survivin knockdown was increased from 3 days to 9 days with slow-NSC and to 5 days with fast-NSC (both $p<0.01$). When the effect of different carriers delivering 50 nM surv-siRNA were compared in the viability studies, it was shown that cancer cells remained in full chemosensitized state to low docetaxel level (0.18 nM) for 9 days and 5 days in the slow- and fast-NSC groups, respectively, *versus* 3 days in the liposome groups and proprietary treatment group ($p<0.01$).

Conclusions. By carefully manipulating the carrier properties to shape the intracellular siRNA kinetics, sustained and controlled siRNA-mediated survivin knockdown can be achieved. This was translatable into longer and more complete sensitization of prostate cancer to docetaxel chemotherapy, allowing the design of more flexible, effective chemo-RNAi-therapy regimens for cancer treatment.